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Cell production gradients in the developing ferret isocortex

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INTRODUCTION

The neural tube is formed from an epithelium composed of an array of columnar cells, and this arrangement imposes a radial geometry on the processes of neuron production and migration. The general pattern of neuron production involves the release of cells from a proliferative zone lining the lumen of the neural tube, and their migration to the external (pial) surface. The cerebral cortex is generated by a region of the forebrain where the radial constraints on neuron migration result in the accumulation of nerve cells in stacks or columns above their site of origin.

Various authors have reported gradients of cortical histogenesis in different species. Studies on spatial gradients show that neuron release starts at a focus and thence spreads across the surface of the cerebral vesicle, within the boundaries of those areas devoted to corticogenesis. The mechanisms determining the location of this focus and the rate of spread of neuron release are not known.

Forebrain neuron production is confined to a brief fetal period (between four and five days in the mouse), indicating a rapid change from proliferation to differentiation within the ventricular germinal layers. Though still a relatively rapid process, neurogenesis in larger mammals requires a longer period for the production of a larger neuron population. The ferret (*Mustela putorius*), is a small carnivore with a relatively large brain and a long gestation period, and the duration of the proliferative phase of forebrain development permits the resolution of events and stages of development which are more compressed in the mouse.

This paper reports a study of two fetal ages when forebrain neuron production is in progress, surveying the laterodorsal and rostrocaudal gradients of neurogenesis by recording the number of cells found in radially arranged sample sites at suitable locations across the cortical plate.

MATERIALS AND METHODS

Tissue preparation

A colony of ferrets was maintained on a small carnivore diet on a 14:10 hours light/dark cycle. Females were housed in a group cage until mating, then individually during pregnancy. Mating was kept to a reasonably short duration (one day) as a compromise between the need for accurately timed gestation and minimal risk of false pregnancy. The gestation period for the ferret is 42 days, and gestations were timed from the day after mating (Gulamhusein & Beck, 1981). The ages selected for study were 22, 29 and 36 days postconception. On the required day of gestation, pregnant females were anaesthetised with Sagatal and the embryos removed surgically and perfused intracardially with physiological saline, followed by Bouin's solution,

at room temperature. The embryos were soaked in Bouin's solution for 4–6 hours, after which the brains were removed and left soaking in Bouin's overnight. The brains were then transferred to 70% alcohol, dehydrated, blocked in paraffin wax and sectioned in the coronal plane on a rotary microtome at a thickness of 6 μm. Serial sections were mounted in sequence on numbered slides, and stained in haematoxylin and eosin before being permanently mounted under coverslips with DPX. Slides were labelled according to the gestation date: for exampe, FE 29 indicated 29 days postconception, and this convention was used throughout the paper. Inspection of the range of embryos available indicated that cortical plate production had not commenced at FE 22, so this stage was not examined further. By FE 29, accumulation of neurons within the cortical plate was in progress over the entire surface of the cerebral hemisphere, and thus a comparison between different areas was possible. By FE 36 it appeared that neuron production was nearing completion at the rostral pole of the cerebral vesicle, while still in progress caudally. These two ages were selected as representing significantly separated phases of neurogenesis, and were analysed further.

Selection of survey levels

Three sets of serial sections were prepared at each of the selected ages, and one optimal set, as judged by symmetry of plane of section and quality of staining, was chosen for further study. A set of serial sections was examined, and the first section exhibiting a clear passage connecting the lateral ventricles (the interventricular foramen), was marked as the 'midsection' at each age. This was designated Level II in subsequent surveys. Levels I and III were determined by counting the number of sections between Level II and the rostral and caudal poles of the cerebral hemisphere, marking the sections located midway along each interval. The relationship between Levels I, II and III is illustrated in Figure 1 *a* using sections from the FE 36 specimen.

Selection of survey sites

Narrow radial sectors at lateral, laterodorsal and dorsal locations were chosen at each age; Figure 1 *b* illustrates these locations for Level II in FE 36. Location C was chosen to sample cortical plate production associated with the ventricular layer close to the caudatopallial angle, a site which has been identified as the ventral boundary of the corticogenic surface in the mouse (Smart & McSherry, 1982). Location A sampled cortical plate development at a dorsal location, and was chosen because the curvature of the ventricle in this area provided a dorsal apex which could be located reliably in different sections at each age. Location B was chosen to sample cortical plate growth midway between C and A. The three locations provided a means of assessing any laterodorsal gradients of neuron production at each level, while the three levels monitored rostrocaudal gradients at reproducible locations for each age.

General survey method

A 'survey transect' was prepared by drawing the outlines of cell nuclei found in a narrow radial column, 125 μm wide, extending from the ventricular to the pial surface. Drawings were prepared at a magnification of $\times 400$, using a Leitz drawing tube mounted on the microscope, and reduced photographically for reproduction. These survey transects provided a visual record of the relevant histological development at each location, without the need for numerous photographic montages, and free of extraneous details such as blood vessels.

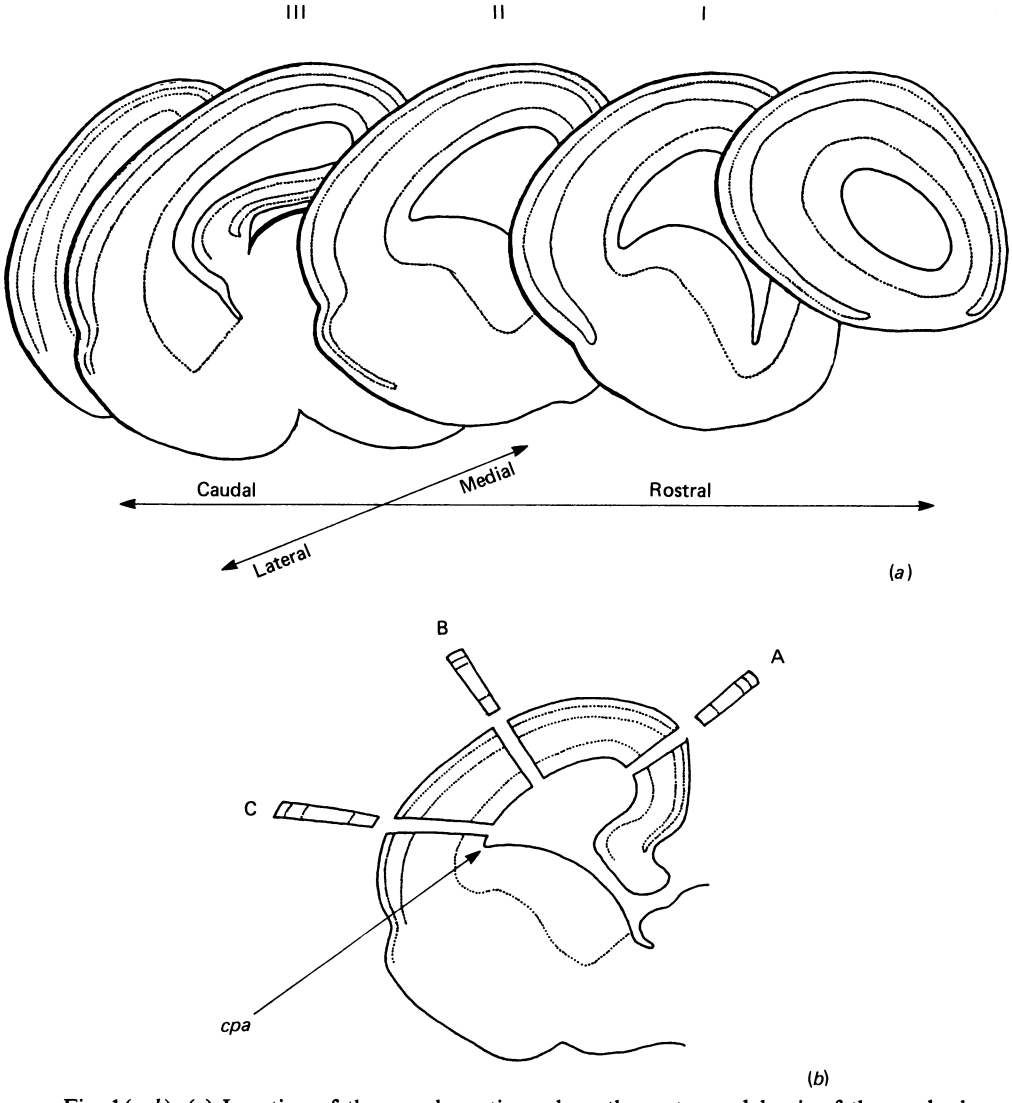
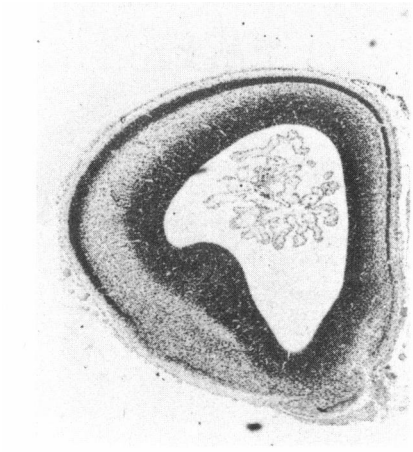
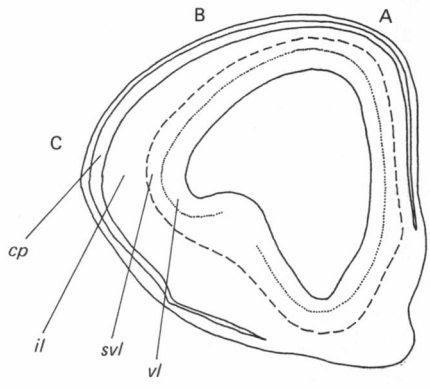


Fig. 1(*a–b*). (*a*) Location of the sample sections along the rostrocaudal axis of the cerebral vesicle. Level II was at the interventricular foramen, Levels I and III were midway between Level II and the rostral and caudal poles respectively. (*b*) Location of the sample sites along the laterodorsal axis. Location C was close to the caudatopallial angle (*cpa*); Location A close to the dorsal apex of the roof of the ventricle and Location B approximately midway along the lateral wall between A and C.

Preparation of cell counts

At a magnification of ×400, the microscope was focused at the mid-depth of each section and, using a drawing tube, cells with nuclear perimeters clearly in focus at that plane within a narrow radial strip 25 μm wide were marked on specially prepared score sheets. The number of cells from the pial surface to the lower border of the cortical plate was scored. These score sheets were counted at a later time, to minimise the effects of observer bias and habituation on the scoring process.





Cortical plate cell counts

Tissue	Ferret					
Age	29					
Level	I					
No.	A	B	C			
1	20	31	45			
2	19	34	39			
3	16	34	45			
4	24	37	46			
5	20	35	37			
6	20	32	34			
7	22	34	45		Mean	S.D.
8	27	37	41	A	21·4	3·37
9	20	32	46	B	33·4	2·75
10	26	28	46	C	42·4	4·37

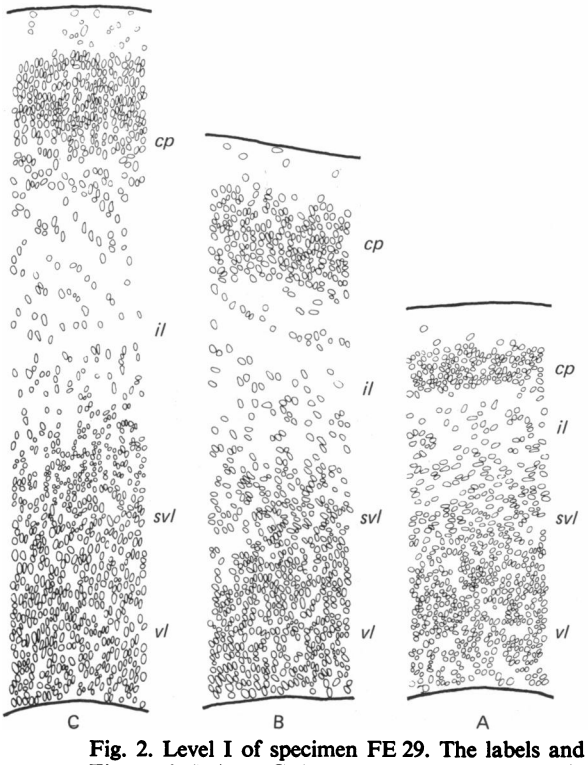


Fig. 2. Level I of specimen FE 29. The labels and abbreviations used here are also used in Figures 3–7. A, B, C show the sample locations. *vl*, ventricular layer; *svl*, subventricular layer; *il*, intermediate layer; *cp*, cortical plate. Table 1 shows the cortical plate cell counts, with means and standard deviations, for each of the sample locations.

Ten radial strips, taken from alternate sections situated rostral and caudal to each particular location, were counted in this manner. Thus, 10 samples were taken from alternate sections adjacent to each of the 18 locations (i.e. A, B and C at Levels I, II and III in specimens FE 29 and FE 36).

Analysis of cell counts

The cell counts were used to prepare a mean cell number for each location. At each level, results for the Locations A and B, and B and C were compared and tested for significant differences using joint variances calculated from the original cell counts, and a standard two-tailed t test (degrees of freedom = 18). A value of $t > 3{\cdot}922$ ($P = 0{\cdot}001$) was taken to indicate that the mean cell numbers were significantly different. This set of comparisons was used to monitor the laterodorsal gradient of cell production present at each level for each age. A second set of comparisons, in which Levels I and II, and II and III were compared for each location, was carried out to monitor the rostrocaudal gradient of cell production. No comparison was made between animals.

RESULTS

Findings are presented in two sections, one dealing with the descriptive surveys of the developing ferret brain, the other with the quantitative surveys of cell production. The terminology used in the descriptive survey is the same as that used in Smart & McSherry (1982) for mouse cortical development.

Development

FE 29 (Length of cerebral hemisphere: 3·9 mm)

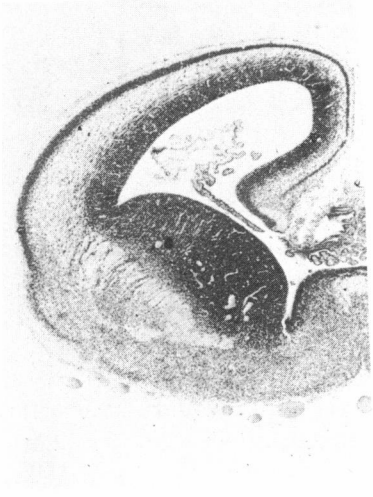
Level I (Fig. 2). A substantial cortical plate, consisting of closely packed, elongated pale nuclei, was present at IC and in a reduced form at IB and IA.

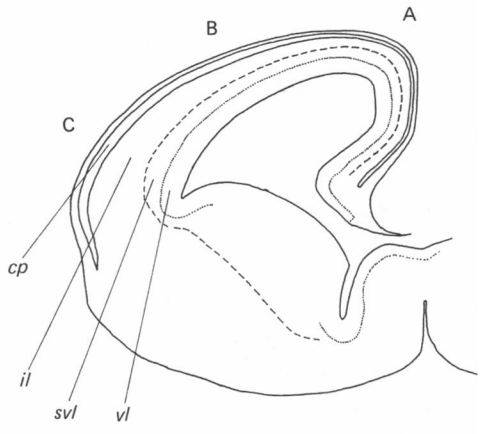
Level II (Fig. 3). The cortical plate, though reduced relative to FE 39(I), was also present at all locations: the plate at IIC was similar to that at FE29(IB), while the plate at IIB was similar to FE29(IA). The cortical plate at IIA was represented by a band of closely packed, elongated nuclei, which was clearly recognisable as a distinct layer.

Level III (Fig. 4). The cortical plate, though reduced further from FE 29(II), was again present at all locations. The relationship noted between II and I above was repeated here between III and II, in that IIIC resembled IIB, while IIIB resembled IIA. The cortical plate at IIIA was reduced compared to IIA, and had a disorganised appearance which suggested that the aggregation process was not as far advanced.

FE 36 (Length of cerebral hemisphere: 4·9 mm)

Level I (Fig. 5). Although occasional tangentially running fibres were found in the FE 29 brain, the ventricular wall at FE 36 was distinguished by the appearance of a new 'striate' layer, which consisted of a substantial band of 'horizontal' or tangential fibres passing through the intermediate layer. The cortical plate was substantially increased relative to FE 29(I) and appeared to have a uniform thickness at all locations. Thus the plate at C, B and A no longer revealed the developmental sequence noted in FE 29. The cells between the lower border of the cortical plate and the upper border of the new striate layer constituted the 'subplate', and probably consisted of a mixture of early formed preneurons and later born cells migrating to more distal levels of the plate. Some of these subplate cells would eventually form part of the lower layers of the cerebral cortex.





Cortical plate cell counts

Tissue	Ferret
Age	29
Level	II

No.	A	B	C		Mean	S.D.
1	16	21	43			
2	11	27	43			
3	15	25	38			
4	15	23	43			
5	13	26	45			
6	13	25	38			
7	14	26	34			
8	11	23	45	A	13·6	1·89
9	12	21	45	B	24·1	2·07
10	16	24	34	C	40·8	4·41

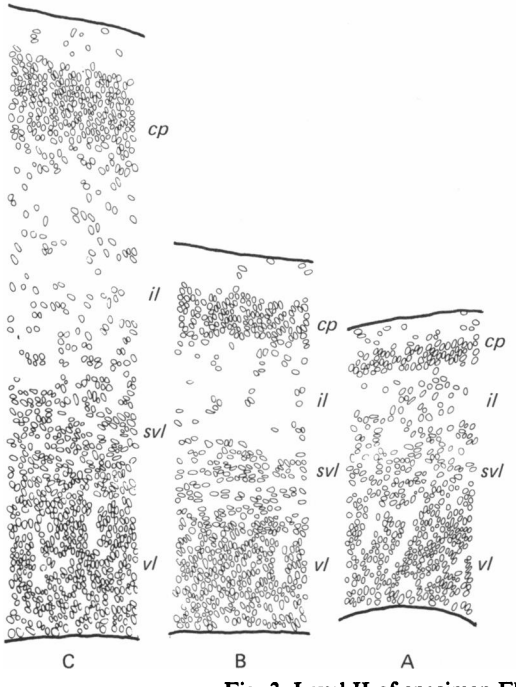
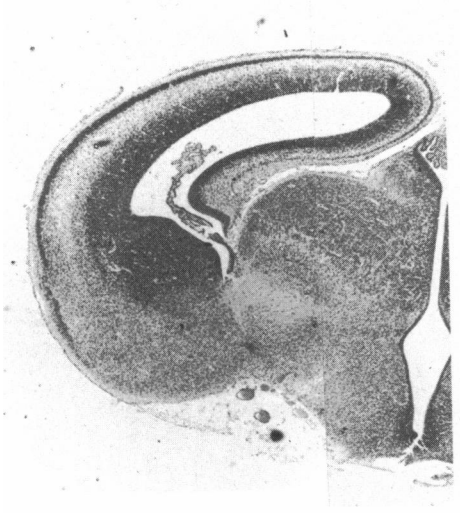
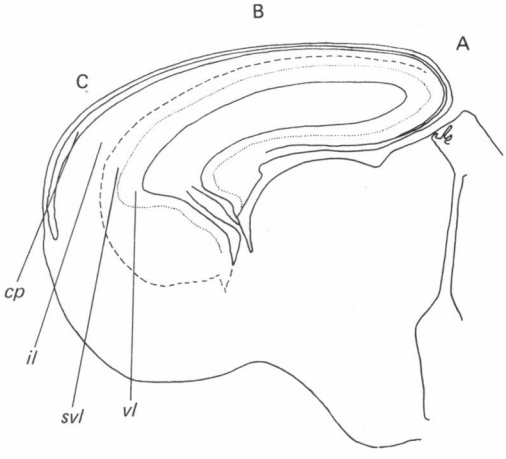


Fig. 3. Level II of specimen FE 29. Abbreviations as in Figure 2.





Cortical plate cell counts

Tissue	Ferret
Age	29
Level	III

No.	A	B	C		
1	13	15	23		
2	13	16	22		
3	12	16	27		
4	13	15	32		
5	19	16	23		
6	14	21	27		
7	16	16	31	Mean	S.D.
8	15	17	29	A 14·5	2·06
9	14	16	26	B 16·4	1·71
10	16	16	22	C 26·2	3·67

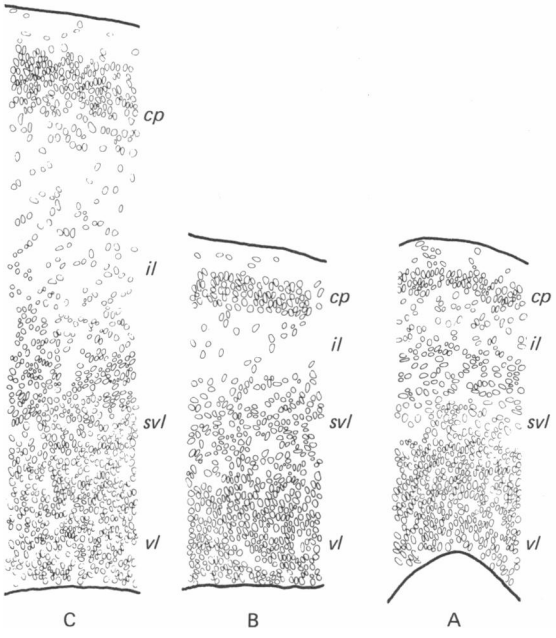
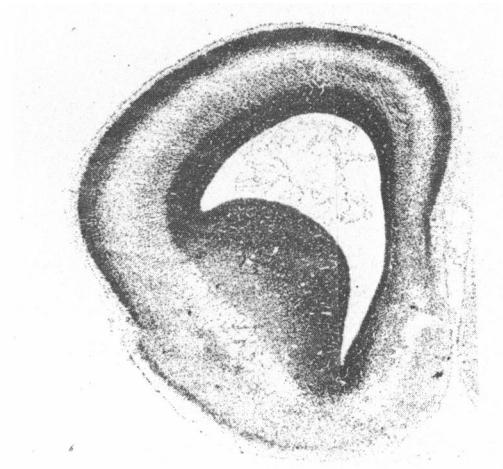
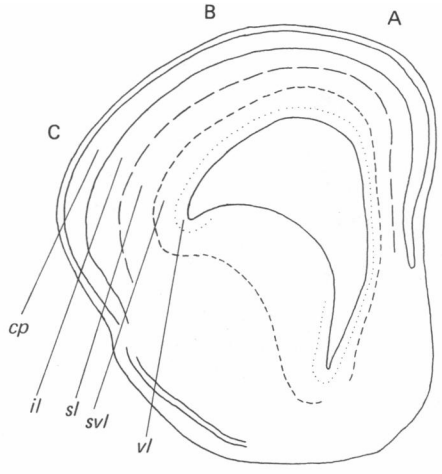
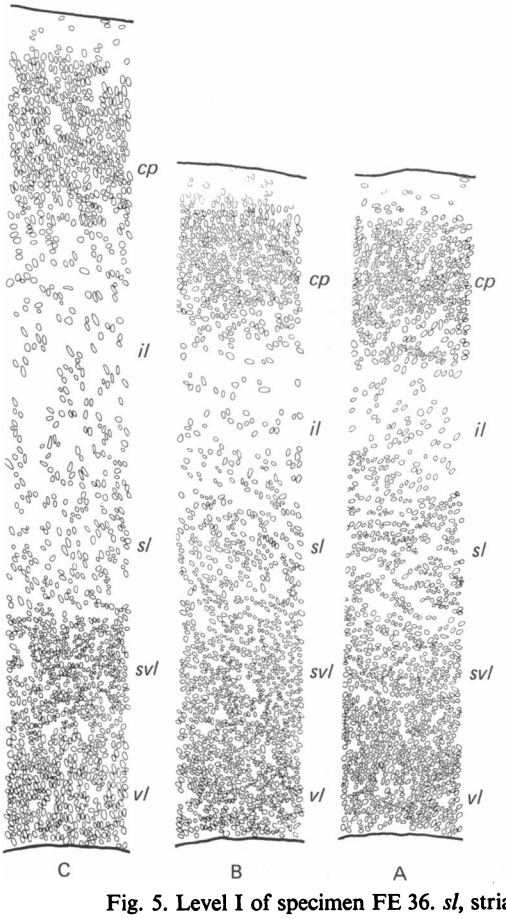


Fig. 4. Level III of specimen FE 29. Abbreviations as in Figure 2.







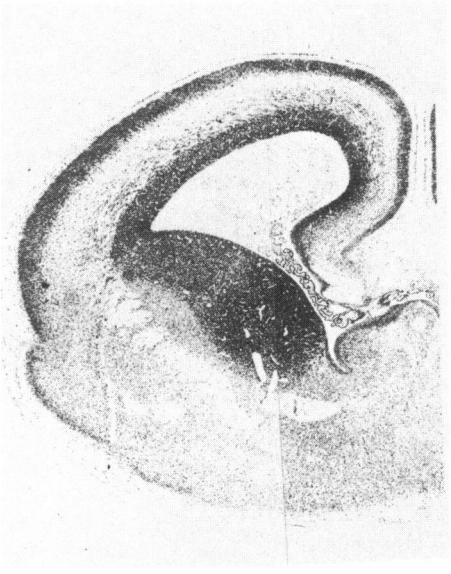
Cortical plate cell counts

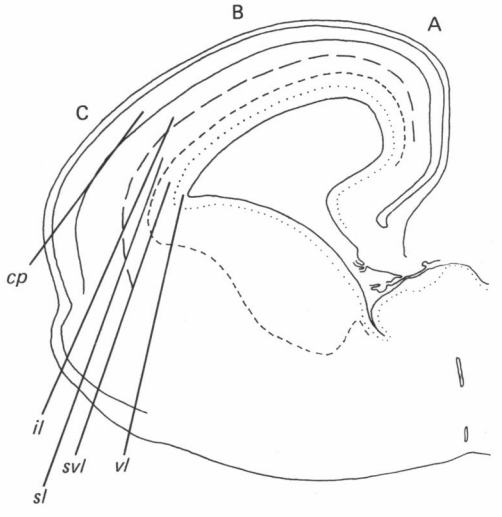
Tissue Ferret
Age 36
Level III

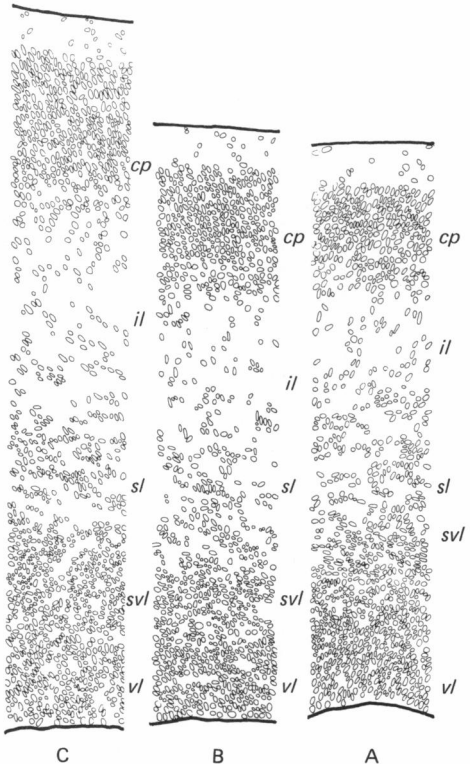
No.	A	B	C
1	57	57	63
2	80	69	75
3	69	69	65
4	70	56	54
5	70	63	52
6	55	59	51
7	62	53	54
8	67	51	59
9	63	70	41
10	66	59	58

	Mean	S.D.
A	65·9	7·18
B	60·6	6·86
C	57·2	9·21

Fig. 5. Level I of specimen FE 36. *sl*, striate layer. Other abbreviations as in Figure 2.





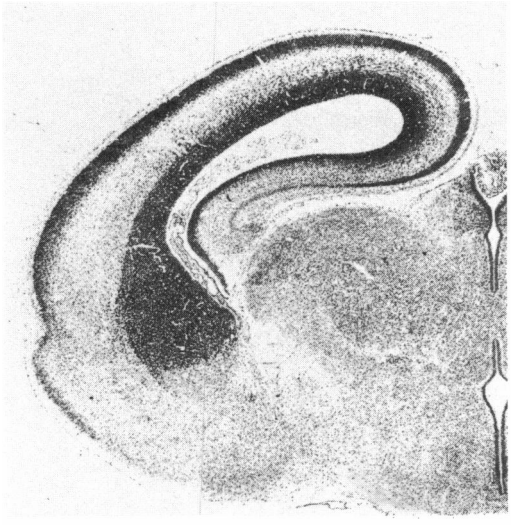


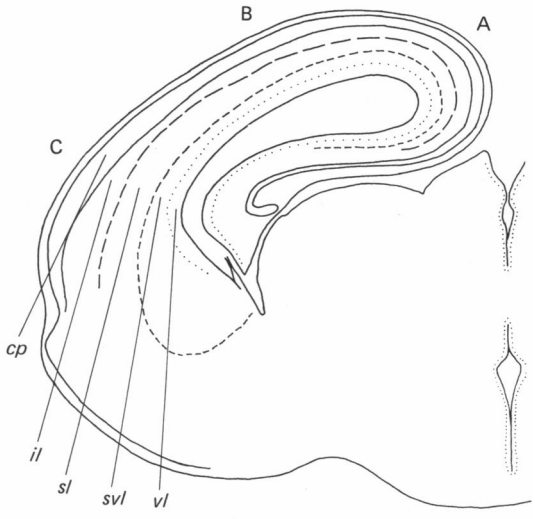
Cortical plate cell count

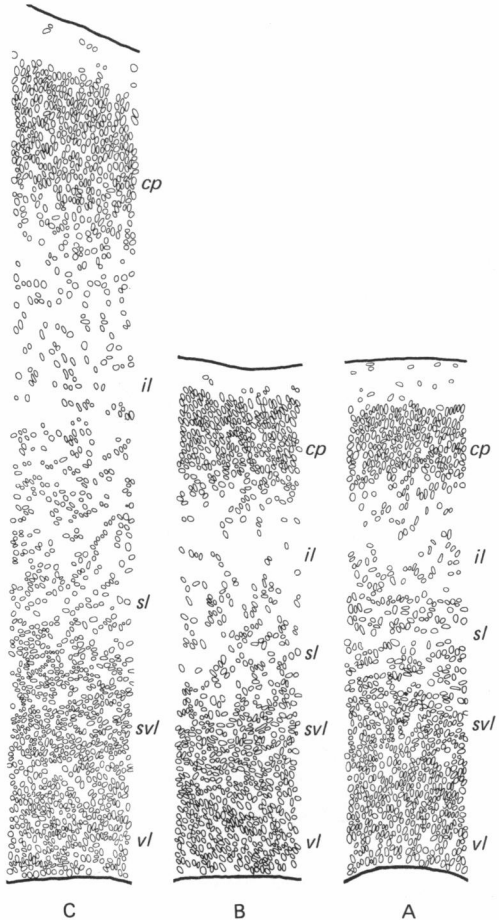
Tissue	Ferret		
Age	36		
Level	I		

No.	A	B	C		
1	46	49	58		
2	45	45	63		
3	40	49	69		
4	47	47	63		
5	36	48	65		
6	51	40	62		
7	37	46	61	Mean	S.D.
8	43	50	55	A 43·5	4·64
9	46	48	67	B 47·4	3·27
10	44	52	54	C 61·7	4·87

Fig. 6. Level II of specimen FE 36. Abbreviations as in Figures 2 and 5.







Cortical plate cell counts

Tissue	Ferret		
Age	36		
Level	II		

No.	A	B	C		Mean	S.D.
1	37	42	63			
2	42	46	68			
3	39	47	59			
4	40	47	59			
5	27	39	54			
6	36	39	60			
7	37	49	66			
8	35	41	58	A	37·1	4·33
9	42	45	57	B	43·6	3·62
10	36	41	47	C	59·8	6·35

Fig. 7. Level III of specimen FE 36. Abbreviations as in Figures 2 and 5.

Table 1. *Comparison of mean cell counts at different sample locations*

Comparisons between A and B and B and C monitor laterodorsal gradients at each level, while comparisons between I and II and II and III monitor rostrocaudal variations at each sample location ('*' beside a specimen pair indicates significance at a level of $P < 0{\cdot}001$).

		FE 29	FE 36
		Laterodorsal	
I			
	A:B	21·4:33·4*	65·9:60·6
	B:C	33·4:42·4*	60·6:57·2
II			
	A:B	13·6:24·1*	43·5:47·4
	B:C	24·1:40·8*	47·4:61·7*
III			
	A:B	14·5:16·4	37·1:43·6
	B:C	16·4:26·2*	43·6:59·8*
		Rostrocaudal	
A			
	I:II	21·4:13·6*	65·9:43·5*
	II:III	13·6:14·5	43·5:37·1
B			
	I:II	33·4:24·1*	60·6:47·4*
	II:III	24·1:16·4*	47·4:43·6
C			
	I:II	42·4:40·8	57·2:61·7
	II:III	40·8:26·2*	61·7:59·8

Level II (Fig. 6). The striate layer was again present at all locations at this level. Although the cortical plate at IIC seemed unchanged relative to IC, B and A both seemed to be reduced relative to the corresponding locations in I.

Level III (Fig. 7). The striate layer was present at all locations. The cortical plate appeared to be similar in depth at A and B, and resembled the corresponding locations in II, while C remained deeper and resembled IIC.

Cell counts

The mean cell numbers from measurements within radially arranged sample columns at Locations A, B and C are presented in Tables accompanying the relevant Figures (Figs. 2–7). These counts show the total number of cells scored from the lower boundary of the cortical plate to the pial surface, and confirmed the impression gained from the descriptive surveys of marked rostrocaudal and laterodorsal gradients of cell accumulation in the cortical plate.

Twelve comparisons were made at each age: Locations A vs B and B vs C at Levels I, II and III (the laterodorsal comparisons in Table 1); I vs II and II vs III at Locations A, B and C (the rostrocaudal comparisons in Table 1). Of these twelve comparisons, nine proved significantly different for FE 29, while only four were significantly different for FE 36.

DISCUSSION

The objective of the present study was to establish a method of surveying the regional variations in neocortical development in the ferret. The method employed depends on certain assumptions about the spatial organisation of neuron production,

and these assumptions deserve further comment. Surveying the brain during embryological development presents many difficulties. In the case of the developing ferret brain, and in particular, the development of its neocortical surface, the problem of measuring comparable regions at different ages is made difficult by the change in size and the scarcity of fixed landmarks. It is necessary to develop a familiarity with the changing morphology of the tissue, and to use experience gained from detailed observation of the growth process in other smaller brains as a guide.

The sampling method chosen utilised a combination of fixed and proportional regions. In serially sectioned brains, the level of the interventricular foramen was chosen as an easily identifiable and relatively fixed anatomical marker. The interventricular foramen was used as Level II in the present study, and Levels I and III were taken as the sections located midway between Level II and the rostral and caudal poles respectively. Thus, at each age, Level II represented a definite anatomical feature that could be easily located, while I and III were proportionally related to it.

The sampling method used reflects a belief in the importance of the columnar organisation of cortical histogenesis (Smart & McSherry, 1982). In so far as the migration of cortical neurons is predominantly radial, and neuron accumulation is thereby constrained within developmental columns, the most appropriate method for monitoring the progress of cortical histogenesis in any mammalian brain would appear to be one which samples narrow radial sectors of the telencephalic wall.

The main findings of the present study are the pronounced gradients of cell production revealed by the descriptive surveys and the regional sampling technique, and this is in accordance with similar gradients reported in mouse, rat, rabbit and cat. Angevine & Sidman (1961) demonstrated that in the mouse, early born neurons lie predominantly in the deep cortex, while later born cells are found in the outermost regions. This classic 'inside-out' pattern of cell accumulation in the cortical plate constitutes a temporal gradient that relates radial depth within the cortex with order of birth. Hicks & D'Amato (1968) in the rat and Fernandez & Bravo (1974) in the rabbit noted that cells of a particular generation are found at deep locations in dorsal cortex and more superficially in lateral cortex. In the light of the general 'inside-out' pattern of cortical formation, lateral cortex is therefore developmentally more advanced than medial.

Studies in the cat (Marin-Padilla, 1978) and mouse (Smart, 1973) describe the progressive growth of the neocortex, from an initial site opposite the internal capsule. In the cat, the progress of cortical plate across the surface of the cerebral vesicle is accomplished in about five days. Measuring both cortical plate depth and distribution of autoradiographically labelled cells in embryonic mouse forebrain, Smart & Smart (1982) and Smart & McSherry (1982) identified a wave of increased neuron birth, consistent with a travelling 'pulse' of neuron production which traversed the cerebral hemisphere in less than two days. McSherry's (1984) survey of cortical plate depth in the ferret revealed rostrocaudal and laterodorsal gradients of plate formation originating from a common focus. The newborn ferret brain reveals a cortical plate of generally uniform cell content throughout, along both the rostrocaudal and the laterodorsal axes, suggesting that the cell production of the ventricular layer tends to even out the earlier regional differences.

The differences in cell production, reflected in the cortical plate cell counts reported above, seem to repeat the same sequence laterodorsally as that observed along the rostrocaudal axis, indicating a common regime of production in both directions. The fact that the different phases of cell production can be traced along

both axes and eventually result in a cortical plate of overall uniform content is strong evidence that the observed gradients represent a true developmental sequence, and is consistent with recent studies of cortical histogenesis in the mouse which present the various phases of neuron production in terms of the activity of radially arranged production units or 'developmental columns', which become active in a controlled sequence across the ventricular layer.

These gradients of cell production have certain implications for experimental studies of brain development in the ferret, which is becoming increasingly used in teratological studies (Beck, 1975; Gulamhusein & Beck, 1981). The effects of cytotoxic agents, which are known to disrupt neuron production, have been studied in the ferret, both histologically and behaviourally (Hadad, Dumas & Burgio, 1974; Hadad & Rabe, 1980). Ferrets treated with methylazoxymethanol acetate have been used as an experimental model of microcephaly, and a variety of anatomical and behavioural abnormalities associated with treatment on different days of gestation has been noted. Treatment at 32 days post conception resulted in the appearance of lissencephaly in the posterior third of the cerebral hemisphere, while the anterior third generally developed cerebral convolutions which approximated to the normal pattern. This tendency to localise the defect in the posterior region of the cerebral hemisphere at this age is consistent with the rostrocaudal gradient of neuron production noted above, in that the anterior hemisphere should be nearer to completing its neuron production, and therefore suffer less from the effect of a toxic agent which disrupts cortical histogenesis. The same consideration applies to the laterodorsal gradient, and one would expect treatment with methylazoxymethanol to have a more severe effect dorsally than laterally, though the behavioural consequences are not easy to predict. However, the anatomical consequences might be a reduction in fissuration in the dorsal and medial cortical surface relative to the lateral cortex.

Treatment with methylazoxymethanol on Day 38 of gestation resulted in a very slight reduction in brain size and cerebral convolutions (Hadad & Rabe, 1980), indicating that the bulk of cortical neuron production has been completed by this age. This is consistent with the estimates of cell numbers in the cortical plate samples presented here for FE 36, in that there is no longer any rostrocaudal variation at Location C (the earliest developed area of cortical plate), nor any laterodorsal variation at Level I, though some variation can be found proceeding caudally and dorsally from these sites.

SUMMARY

The accumulation of cells within the cortical plate was studied in ferrets at two developmental ages. A survey method based on the presumed radial organisation of cortical neuron production was used to sample variations in cell production along the rostrocaudal and laterodorsal axes of the brain. The resulting cell counts confirmed the presence of a gradient of cortical plate formation, with a rostrolateral focus. These findings were discussed in relation to some recent teratological studies on brain development in ferrets, where there has been a lack of normative data.

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